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RESEARCH ARTICLE

The Balkan chamois, an archipelago or a peninsula? Insights from nuclear and mitochondrial DNA

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Abstract

The Balkan chamois (*Rupicapra rupicapra balcanica*) is widespread on the Balkan Peninsula, along mountain massifs from Croatia in the north to Greece in the south and Bulgaria in the east. Knowledge on the genetic structure of Balkan chamois populations is limited and restricted to local studies. Therefore, the main objective of this study was to use nuclear (16 microsatellites) and mitochondrial (partial 376 base pairs control region) markers to investigate the genetic structure of this chamois subspecies throughout its distribution range and to obtain information on the degree of connectivity of the different (sub)populations. We extracted DNA from bone, dried skin and muscle tissue and successfully genotyped 92 individuals of Balkan chamois and sequenced the partial control region in 44 individuals. The Bayesian analysis suggested 3 genetic clusters and assigned individuals from Serbia and Bulgaria to two separate clusters, while individuals from the other countries belonged to the same cluster. Thirty new haplotypes were obtained from partial mitochondrial DNA sequences, with private haplotypes in all analyzed populations and only two haplotypes shared among populations, indicating the possibility of past translocations. The subspecies genetic composition presented here provides the necessary starting point to assess the conservation status of the Balkan chamois and allows the development of conservation strategies necessary for its sustainable management and conservation.

Keywords Conservation · Genetic diversity · mtDNA · Population genetics · *Rupicapra rupicapra balcanica*

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Introduction

Chamois (*Rupicapra* spp.) are medium-sized ungulates that inhabit alpine pastures and rocky areas on the main mountain massifs of both Europe and the Near East (Corlatti et al. 2011) and, exceptionally, the low elevations of river gorges (Papaioannou and Kati 2007), forested and coastal areas (Safner et al. 2019; Kavčić et al. 2020). The current distribution of *Rupicapra* spp. has been shaped by the natural fragmentation of suitable habitats and the resulting constraints on gene flow between different populations (Buzan et al. 2013).

The currently accepted taxonomy of chamois, based on morphological, behavioral and molecular evidence, recognizes two species: the Northern chamois (*Rupicapra rupicapra*) with seven subspecies distributed in the Alps (*R. r. rupicapra* and *R. r. cartusiana*), the Balkans (*R. r. balcanica*), the Tatras (*R. r. tatrica*) and the Carpathians (*R. r. carpatica*), in western Asia (*R. r. asiatica*) and the Caucasus (*R. r. caucasica*) (Anderwald et al. 2020); and the Southern chamois (*Rupicapra pyrenaica*), which includes three geographically isolated subspecies on the Cantabrian Massif (*R. p. parva*), the Pyrenees (*R. p. pyrenaica*) and the central Apennines (*R. p. ornata*) (Herrero et al. 2020).

Over the past two decades, the genus *Rupicapra* has been the subject of numerous genetic studies in which, depending on the scope of the study, different markers were used. Microsatellites have been a useful tool in studies of population structure (Papaioannou et al. 2019; Soglia et al. 2010; Šprem and Buzan 2016), phylogeography (Pérez et al. 2002; Rodríguez et al. 2010) or conservation (Buzan et al. 2013; Crestanello et al. 2009; Markov et al. 2016). Complete mitogenome (Pérez et al. 2014; Iacolina et al. 2021) or partial regions of maternally inherited mitochondrial DNA (mtDNA) have been used to study population genetics, systematics and evolution (Hammer et al. 1995; Rodríguez et al. 2007; Rodríguez et al. 2009; Buzan et al. 2013; Šprem and Buzan 2016; Pérez et al. 2017b; Moravčíková et al. 2019). Nuclear markers such as autosomal introns (Pérez et al. 2017a) or the melanocortin-1 receptor gene (MC1R; Pérez et al. 2013), which is related to the coloration pattern of chamois fur, have been used to study the effects of historical and evolutionary events on the diversification of *Rupicapra* spp., whereas the Y chromosome use has been rare and limited to phylogenetic studies, due to the methodological difficulties associated with the marker (Pérez et al. 2011).

The effects of historical and evolutionary events on chamois diversification and taxonomy are still under discussion. Previous phylogenetic findings based on mtDNA suggest the presence of three major mitochondrial lineages within the genus *Rupicapra* which correspond to the geographic distribution of the species and are referred to as the West (*R. p. parva* and *R. p. pyrenaica*), Central (*R. p. ornata* and *R. r. cartusiana*) and East (the six remaining subspecies including *R. r. balcanica*) (Rodríguez et al. 2009; Rodríguez et al. 2010; Pérez et al. 2014). Although Balkan chamois has been included in several phylogenetic studies that used both nuclear and mitochondrial markers (Rodríguez et al. 2009; Rodríguez et al. 2010; Pérez et al. 2017a; Pérez et al. 2017b), it remains one of the less-studied Northern chamois subspecies (Kati et al. 2020). Current knowledge on the genetic diversity and structure of the Balkan chamois population is limited and restricted to regional-local studies (e.g. Bulgaria—Markov et al. 2016; Croatia and Bosnia and Herzegovina—Šprem and Buzan 2016; Greece—Papaioannou et al. 2019). The distribution of the Balkan chamois is patchy and covers only parts of the massifs and mountain chains across the countries that form its range (Fig. 1b). According to Buzan et al. (2013), the subspecies dispersals is a legacy of Quaternary glacial-interglacial dynamics when many taxa were trapped in relatively small refugial areas. This scenario supports the combined results from the major histocompatibility complex (MHC) and mtDNA sequences that the last glaciation produced a demographic decline and small subunits in chamois populations (Mona et al. 2008). The subspecies' low rates of colonization and reduced gene flow between isolated populations may result in genetic differentiation due to the inbreeding effect and a loss of allelic variants as a consequence of genetic drift (Willi et al. 2006). Reduced genetic diversity in small and isolated populations might, in turn, cause negative impacts on fitness, resulting in decreased effective population size and, eventually, increase the probabilities of extinction (Pelletier et al. 2019). Other threats to the Balkan chamois survival are considered to be poaching (Papaioannou and Kati 2007), introductions of other chamois subspecies, mostly Alpine chamois (Iacolina et al. 2019), forest succession (Kavčić et al. 2019), road infrastructure (Kati et al. 2020), intensive livestock grazing, predation, unsustainable hunting and natural events (Šprem and Buzan 2016). Due to these threats**,** the Balkan chamois is protected by Annexes II and IV of the European Union Habitats Directive 92/43/EEC (OJ L 206, 22.7.1992) and Appendix III of the Bern Convention (OJ L 38, 10.2.1982). The conservation and management status within the different national legislations varies between countries (members and non-members of the EU) and depends on the degree of the local communities' interest in the conservation of the subspecies (Anderwald et al. 2020). Considering the demographic independence of populations, conservation objectives should be focused on defining conservation units such as management (MUs) or evolutionarily significant units (ESUs), based on a combination of molecular, morphological, ecological, behavioral and biogeographic information that can be combined to identify populations that need to be managed together (Funk et al. 2013).

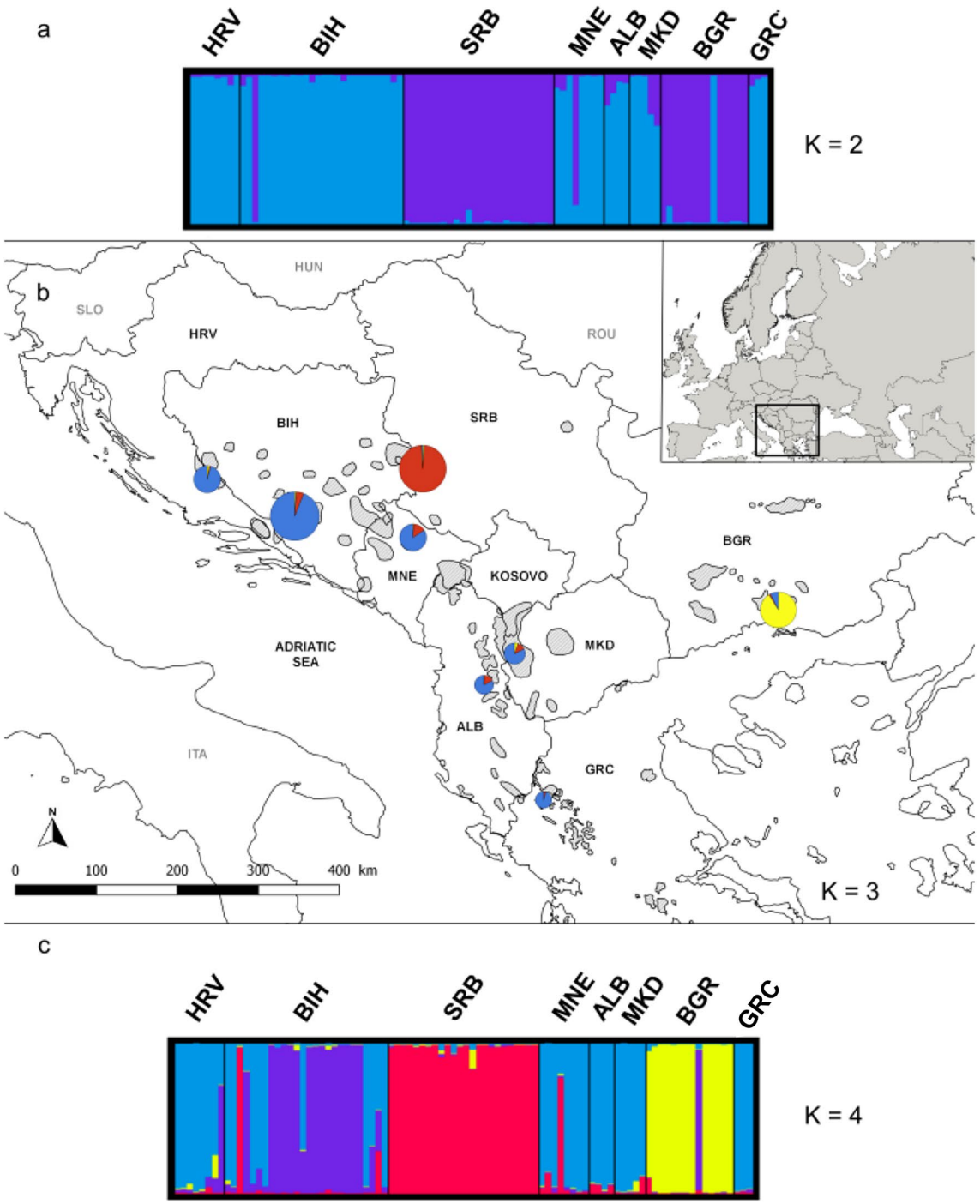

Fig. 1 Analyses of the genetic structure of Balkan chamois populations across the Balkan Peninsula. STRUCTURE admixture model results based on sixteen loci with correlated allele frequencies for (a) K = 2, (b) K = 3 and (c) K = 4. **a** Assignment of individual genotypes of Balkan chamois to clusters K = 2 as inferred by STRUCTURE. **b** Geographic distribution of the Balkan chamois genotypes. The distribution range map (in transparent grey) was constructed using shape data downloaded from the IUCN Red List of Threatened Species. Each pie chart indicates the geographic location of the sampled population. The size of the pie charts indicates the number of samples collected from a locality. Different colors of the pie chart indicate proportions of respective genetic clusters per individual Q (in %). **c** Assignment of individual genotypes of Balkan chamois to clusters K = 4. *HRV* Croatia, *BIH* Bosnia and Herzegovina, *SRB* Serbia, *MNE* Montenegro, *ALB* Albania, *MKD* North Macedonia, *BGR* Bulgaria, *GRC* Greece

The main objective of this study was to gather information on the genetic diversity and population structure of Balkan chamois along its distribution range to support the development of management and conservation strategies. With this aim, we used microsatellite genotypes and a partial region of mtDNA (Control Region—CR) to address questions regarding the influence of geographical isolation on the genetic diversity of Balkan chamois populations.

Materials and methods

Samples collection and DNA extraction

A total of 101 specimens of Balkan chamois were collected from 2011 to 2018 at sites throughout its distribution range (Fig. 1b). Of the total number of specimens collected, complete genotypes were obtained for 92 individuals, while mtDNA CR was successfully sequenced in 44 individuals (Table S.1). For population-based analyses, Balkan chamois individuals were divided into eight groups based on country of origin: Croatia (HRV), Bosnia and Herzegovina (BIH), Serbia (SRB), Montenegro (MNE), Albania (ALB), North Macedonia (MKD), Bulgaria (BGR), and Greece (GRC). For mtDNA analyses, we collected 56 additional samples belonging to other chamois subspecies (*R. p. pyrenaica*, *R. p. ornata*, *R. r. rupicapra*, *R. r. tatrica*, *R. r. carpatica*). Samples (bones, dried skin, and muscle tissue) were obtained from hunted and naturally dead animals, remains of poached individuals, and specimens from hunting museum collections. Muscle samples were preserved in 96% ethanol and stored at -80 °C until extraction.

We extracted DNA from muscle (N = 69) and skin (N = 1) samples using the commercial peqGOLD Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH) following the manufacturer´s protocol in a volume of 150 µL. DNA from bone samples (N = 22) was extracted using 400 mg bone powder following the procedure described in Buzan et al. (2020) using the QIAamp DNA Micro Kit (Qiagen) and a final volume of 100 µL.

DNA concentrations were measured with Qubit® dsDNA BR Assay Kit (Invitrogen) on a 3.0 Qubit Fluorimeter (Life Technologies).

Microsatellite amplification and genotyping

Twenty microsatellites were amplified using PCR multiplex sets already screened in studies of chamois (Zemanová et al. 2011; Buzan et al. 2013; Šprem and Buzan 2016). We used KAPA2G Fast Multiplex Mix and protocol (KAPA Biosystems Roche) to amplify the target regions. If the multiplex was unsuccessful, we repeated the analysis with single-locus PCR. Each reaction contained 6.25 µL of KAPA2G Fast Multiplex Mix, 1.7 µL primers (forward fluorescent-labeled) at various concentrations (Table S.2), 2.5 µL of DNA and ddH_2O for a final volume of 13 µL. In a few tissue and bone samples, the quality of DNA was low, so we used a volume of 5 µL of genomic DNA and reduced the amount of water for the same final volume of 13 µL. Fragment analysis was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems) using the GeneScan LIZ500 (-250) Size Standard (Applied Biosystems). Microsatellite genotypes were examined using Gene Mapper v. 4.0 software (Applied Biosystems).

Mitochondrial DNA sequencing

The partial CR of the mtDNA was amplified using the primers and protocol described in Rodríguez et al. (2010). Amplicons were visualized on a 2% agarose gel and purified with ExoSAP-IT PCR product cleanup reagent (Applied Biosystems). Forward and reverse PCR products were sequenced with the ABI BigDye terminator mix v3.1 (Applied Biosystems) followed by electrophoresis in an ABI SeqStudio Genetic Analyzer (Thermo Fischer Scientific).

Microsatellite data analysis

We used the Expectation–Maximization (EM) algorithm implemented in FreeNA (Chapuis and Estoup 2007) to estimate null allele frequencies for each microsatellite locus, since they may cause a significant heterozygote deficit and deviation of populations from the Hardy–Weinberg equilibrium (HWE). Loci that had high estimates of null allele frequencies were excluded from further analysis. The same software was used to calculate the overall genetic differentiation within the dataset, the global F_{ST} value (Chapuis and Estoup 2007).

Loci without null allele frequencies in each sampling population were tested for deviations from HWE with the Markov chain method with 10,000 dememorization steps, 500 batches and 10,000 subsequent iterations in GENEPOP ver. 4.7.2 (Rousset 2008). The exact test based on a Markov chain method implemented in GENEPOP was used to analyze pairwise linkage disequilibrium (LD) among all pairs of loci across all populations. A sequential Bonferroni procedure (Holm 1979) was applied to correct the effect of multiple comparisons tests using adjust p-values function implemented in R ver. 4.0.5 package stats (R Core Team 2020).

Genetix ver. 4.05.2 (Belkhir et al. 1996–2004) was used to calculate the mean number of alleles, observed (H_O) and expected (H_E; Nei 1978) heterozygosities for each locus in all populations as well as the inbreeding coefficient (F_{IS}) and its confidence intervals. We estimated the allelic richness in each population using the rarefaction procedure implemented in FSTAT ver. 2.9.3.2 (Goudet 2001). The number

of private alleles was estimated using GenAlEx ver. 6.502 (Peakall and Smouse 2012). The genetic differentiation between all pairs of populations (pairwise F_{ST}) was estimated using the hierfstat 0.5–7 package (Goudet 2005) in R. The respective p-values were calculated with the same package using 100 bootstraps over loci for each population pair.

STRUCTURE ver. 2.3.4. (Pritchard et al. 2000) was used to assign the Balkan chamois individuals to the most likely number of genetic clusters (K). We performed ten independent runs for each K between 1 and 10 under a model assuming admixture and correlated allele frequency with a burn-in of 10^5 steps and a run length of 10^6 Markov chain Monte Carlo (MCMC) iterations. Structure Harvester (Earl and vonHoldt 2012) was used to compare the average estimates of the likelihood of the data, *ln*[Pr(X|K)] for each value of K and to apply the ad hoc summary statistic ΔK developed by Evanno et al. (2005) to estimate the most likely K. The same software was used to generate graphs for the mean log posterior probability of the data (mean ± SD). The results of replicated runs for each value of K were combined using the Greedy algorithm in CLUMPP ver. 1.1.2 (Jakobsson and Rosenberg 2007) and the summary outputs were displayed graphically using DISTRUCT ver. 1.1 (Rosenberg 2004). The modal cluster membership for each individual in each sampled area from the run with the highest log-likelihoods was plotted on a map using QGIS ver. 2.18.21 (QGIS Development Team 2018). The Photo Scape X software (MOOII Tech) was used for image processing.

Isolation by distance (IBD) between pairs of Balkan chamois populations was tested using the package adegenet 2.0.0 (Jombart 2008) in R. A Mantel test was applied to test the correlation between genetic distance (Edwards 1971) and geographic distances matrices.

We excluded Albania (n = 4) and Greece (n = 3) samples from all population-based analyses because of the small sample size and included them only in the individual-based analyses.

Mitochondrial data analysis

The mitochondrial sequences were manually checked and assembled in FinchTV ver. 1.5.0 (Geospiza Inc.). The 100 new sequences of CR were aligned using the ClustalW algorithm implemented in MEGA X ver. 10.0.5 (Kumar et al. 2018) together with 109 sequences retrieved from GenBank (accession numbers AM279274-279275, EU887481-EU887488, GU951843–GU951916, KC594557-KC594572, KP730619-KP730627; Table S.3). The final alignment consisted of 376 base pairs (bp) and was used to generate haplotypes in DnaSP ver. 5.0 (Librado and Rozas 2009). All newly generated haplotypes were submitted to GenBank (accessions MT746066–MT746095).

Evolutionary relationships between haplotypes were analyzed by a Median-Joining network (Bandelt et al. 1999) constructed with NETWORK ver. 5.0 (Fluxus Technology Ltd.). The weights of characters' value were set to 10, while the parameter epsilon, which specifies a weighted genetic distance to the known sequences in the dataset, was set to 0 to obtain a sparse spanning network.

Results

Within-population genetic diversity

Of the twenty microsatellites chosen for the analysis of the Balkan chamois population structure, locus MAF214 could not be amplified in 12 individuals while loci SY58 and SR-CRSP-9 showed the presence of null allele (Table S.4) and significantly deviated from HWE. These loci were therefore excluded, together with INRA121 that was monomorphic. Of the total 101 samples collected, complete genotypes were obtained for 92 individuals. We re-amplified approximately 10% of the genotypes suspected of allelic dropout and all genotypes were confirmed. The set of sixteen microsatellite loci yielded a total of 116 alleles in the eight Balkan chamois populations, ranging from 2 (ETH10, SR-CRSP-6) to 11 (BOBT24) with an average of 7.25 alleles per locus (Table S.2).

The within-population genetic diversity of the six Balkan chamois populations is shown in Table 1. The HRV and BIH populations showed significant deviation from HWE based on the exact tests in Genepop ($p < 0.05$). After applying sequential Bonferroni correction, the HRV population deviated from HWE for locus BM1258 ($p = 0.04$) while the BIH population deviated significantly ($p < 0.01$) from HWE for loci SY434, SY259 and SR-CRSP-6. The F_{IS} values varied between -0.019 (SRB) and 0.198 (MKD). The sequential Bonferroni correction applied to the linkage disequilibrium test showed a significant value only for locus CSSM66 ($p < 0.05$).

The lowest allelic richness (AR) was detected in BGR (2.522) while HRV population had the highest values (3.187). The observed number of alleles (A) across microsatellite loci ranged from 3.187 in MKD to 5.250 in BIH. All populations had 5 private alleles with exception of SRB that had none, and MNE that had only 3. The H_O varied from 0.489 in the MKD population to 0.605 in the MNE population. The H_E ranged from 0.519 (BGR) to 0.655 (HRV). SRB was the only population that showed an excess of heterozygotes.

Table 1 Genetic diversity of sixteen microsatellite markers in five Balkan chamois populations

Country /population	N	$H_O \pm SD$	$H_E \pm SD$	F_{IS} (IC 95%)	HWE	A	AR	N_{pr}
Croatia (HRV)	8	0.589 ± 0.212	0.655 ± 0.141	0.174 (− 0.077–0.236)	0.007*	4.188	3.187	5
Bosnia and Herzegovina (BIH)	26	0.546 ± 0.250	0.615 ± 0.172	0.133 (0.034–0.187)	0.000**	5.250	2.941	5
Serbia (SRB)	24	0.570 ± 0.212	0.548 ± 0.197	-0.019 (− 0.129–0.045)	0.979^{ns}	3.750	2.595	0
Montenegro (MNE)	8	0.605 ± 0.235	0.619 ± 0.179	0.092 (− 0.113–0.103)	0.203^{ns}	4.250	3.109	3
North Macedonia (MKD)	5	0.489 ± 0.343	0.530 ± 0.213	0.198 (− 0.213–0.198)	0.548^{ns}	3.187	2.709	5
Bulgaria (BGR)	14	0.520 ± 0.213	0.519 ± 0.195	0.035 (− 0.147–0.112)	0.246^{ns}	3.812	2.522	5

N—number of samples; H_O—observed heterozygosity; H_E—expected heterozygosity; SD—standard deviation; F_{IS}—inbreeding coefficient; IC 95%—95% Confidence Interval; HWE—Hardy–Weinberg equilibrium (after Bonferroni adjustment p values: ^{ns}—non—significant value; *—significant at $p < 0.05$; **—significant at $p < 0.01$); A—average number of alleles; AR—allelic richness; N_{pr}—number of private alleles

Genetic variability among populations

The global F_{ST} value for the six populations was 0.184 with a 95% confidence interval significantly different from zero (CI = 0.149 – 0.223). The lowest pairwise F_{ST} value was observed between HRV and MNE populations (0.084), while the highest value was found between SRB and BGR (0.292) populations. Pairwise F_{ST} values between BGR and all other analyzed populations were significant (Fig. S.1). Significant values were found between SRB and other populations except MNE, and between BIH and MKD.

The STRUCTURE results for K = 2 grouped the populations HRV, BIH, MNE, ALB, MKD, GRC, as well as one individual from BGR, while the other cluster contained individuals from BGR and SRB populations and one individual from BIH population (Fig. 1a). According to the Evanno method, the best model of population assignment was with three clusters (Fig. S.2). The STRUCTURE analysis for K = 3 assigned individuals from SRB and BGR to two private clusters while individuals from the other analyzed populations belonged to one common cluster (Fig. 1b). A further increase to K = 4 indicated the divergence of BIH from the other Balkan chamois populations (Fig. 1c).

Applying the Mantel test across the six populations of Balkan chamois, no significant positive relationships were found between genetic Edwards' and geographical distances ($p = 0.375$, $r = 0.082$; Fig. 2).

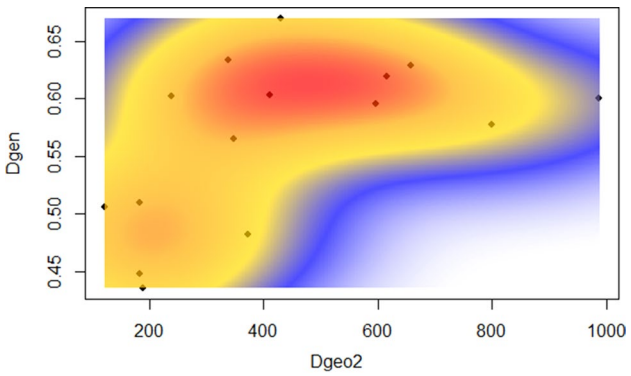

Fig. 2 Correlation analysis between pairwise Edward's genetic distances (Dgen) and the geographic distances (Dgeo; km). The colours represent the relative density of the points, with warmer colours indicating higher density, while the points represent the Edwards genetic distances between the six analysed Balkan chamois populations plotted against the Euclidean geographic distances for the same populations (HRV, BIH, SRB, MNE, MKD, BGR)

Mitochondrial DNA variation

MtDNA variation analysis was performed on a 376 bp sequence of CR. For the analysis of mtDNA diversity and the construction of the haplotype network, two datasets were used, one containing only individuals of Balkan chamois (Table S.1) and the second containing all subspecies analyzed in this study together with GenBank sequences (Table S.3). For the dataset containing only Balkan chamois individuals (44 sequences), the total haplotype and nucleotide diversity were 0.913 and 0.031, respectively (Table 2). The ALB population showed the highest values of haplotype (1.000) and nucleotide (0.030) diversity while the lowest were detected in BGR (Hd = 0.464; $\pi = 0.009$).

The second dataset consisted of 100 new CR sequences, which were aligned together with 109 sequences from GenBank. A total of 30 new haplotypes of CR were defined and deposited in GenBank under Accession numbers MT746066—MT746095 (HRCR17—HRCR46). Of these, 15 were found in Balkan chamois populations, 2 were shared

Table 2 Diversity of partial CR of Balkan chamois populations

Population	N	h	S	Hd ± SD	π ± SD
HRV	5	2	11	0.700 ± 0.218	0.015 ± 0.005
BIH	10	3	22	0.778 ± 0.091	0.026 ± 0.004
SRB	7	2	12	0.476 ± 0.171	0.015 ± 0.005
MNE	4	1	15	0.500 ± 0.265	0.020 ± 0.011
ALB	3	3	17	1.000 ± 0.272	0.030 ± 0.013
MKD	3	2	10	0.667 ± 0.314	0.017 ± 0.008
BGR	8	2	13	0.464 ± 0.200	0.009 ± 0.006
GRC	4	3	20	0.833 ± 0.222	0.028 ± 0.010
Total	44	18	48	0.913 ± 0.023	0.031 ± 0.003

N number of sequences used for analysis, *h* number of haplotypes, *S* number of segregating sites, *Hd* haplotype diversity, *π* nucleotide diversity, *SD* standard deviation, *HRV* Croatia, *BIH* Bosnia and Herzegovina, *SRB* Serbia, *MNE* Montenegro, *ALB* Albania, *MKD* North Macedonia, *BGR* Bulgaria, *GRC* Greece

between Balkan and Tatra chamois, 1 was shared between Balkan and Alpine chamois, while Alpine chamois itself had 8 and Carpathian chamois 3 haplotypes. Two haplotypes were identified in the Southern chamois (Table S.3). Alignment of 209 individual sequences revealed 136 segregating sites and the total number of mutations was 143.

In the network containing only Balkan chamois individuals (Fig. 3a), all eight sampled populations had private haplotypes, while two haplotypes (HRCR9 and HRCR20) were present in multiple populations. One individual of Balkan chamois from the HRV population was assigned the haplotype HRCR29, while others shared the haplotype HRCR9 with individuals from the BIH and BGR populations. Similarly, one individual from the HRV population shared haplotype HRCR20 with individuals from the SRB and MNE populations.

In the median-joining network obtained from 209 individuals (Fig. 3b) three haplotypes were detected where *R. r. balcanica* shared identical sequences with *R. r. rupicapra* (HRCR9) and *R. r. tatrica* (HRCR20 and HRCR38) subspecies (see Table S.3 for haplotypes information).

Discussion

Our results revealed the existence of three genetically and geographically distinct populations of Balkan chamois. Genetic differentiation was most pronounced in BGR and SRB populations while mtDNA results revealed private haplotypes in all eight sampled populations. The increased divergence between Balkan chamois populations and the existence of unique haplotypes could be a result of historical (post-glacial colonization processes) and recent events (anthropogenic impacts). The long-term persistence of subspecies depends on sufficient genetic diversity to adapt to a variable environment, understanding the genetic diversity and structure of subspecies is thus important for taking effective management and conservation action (Souza-Shibatta et al. 2018). The use of molecular markers, in this case microsatellites and mtDNA, provided important information on the genetic diversity and evolutionary history of the Balkan chamois population. Based on our results, we recommend that spatial populations should be considered MUs, in order to ensure effective management and conservation of this subspecies.

Genetic diversity

We excluded four loci from the final dataset due to either poor amplification, high percentage of null alleles or monomorphism. Two of the excluded loci, SY58 and INRA121, presented the same issues in other studies (Buzan et al. 2013; Šprem and Buzan 2016), therefore we recommend excluding these two loci in future studies of *Rupicapra* subspecies.

According to Rodríguez et al. (2010), the reason why loci (SY434, SY259, and SR-CRSP-6) in the BIH population diverged significantly from the HWE could be due to the genetic characteristics of the population (immigrants, Wahlund effect) rather than locus systematical deviation from the HWE. Since there was no deviation of these loci from HWE in other populations, we retained them in all subsequent analyses. Concordantly with our results, in the study by Šprem and Buzan (2016), the population Prenj from Bosnia and Herzegovina showed significant deviation from HWE based on exact tests and significant positive values of F_{IS}, but the deviation from HWE remained non-significant after Bonferroni correction.

The degree of intraspecific genetic diversity is an important criterion for characterizing conservation units and identifying populations to be prioritized for protection (Bonin et al. 2007). Natural selection operates with the genetic raw material of the population (Funk et al. 2013) to enable adaptation to environmental change and, eventually, evolution (Bonin et al. 2007). The interaction of genetic and demographic factors leads to a loss of intraspecific genetic diversity, especially in small and isolated populations which are threatened by inbreeding depression and thus increasing the extinction probability (Bonin et al. 2007). The Balkan chamois populations' genetic diversity analyzed in this study (Table 1) is low when compared with values reported in the study of Rodríguez et al. (2010). Here we report values of allelic richness ranging from 2.522 to 3.187, whereas Rodriguez et al. (2010) observed an allelic richness of 3.74 for the nine individuals of Balkan chamois analyzed. This difference might be due to the higher number of microsatellites used by Rodríguez et al. (2010). A similar situation was found in a study on the population genetics of Alpine chamois (Soglia et al. 2010), which showed higher

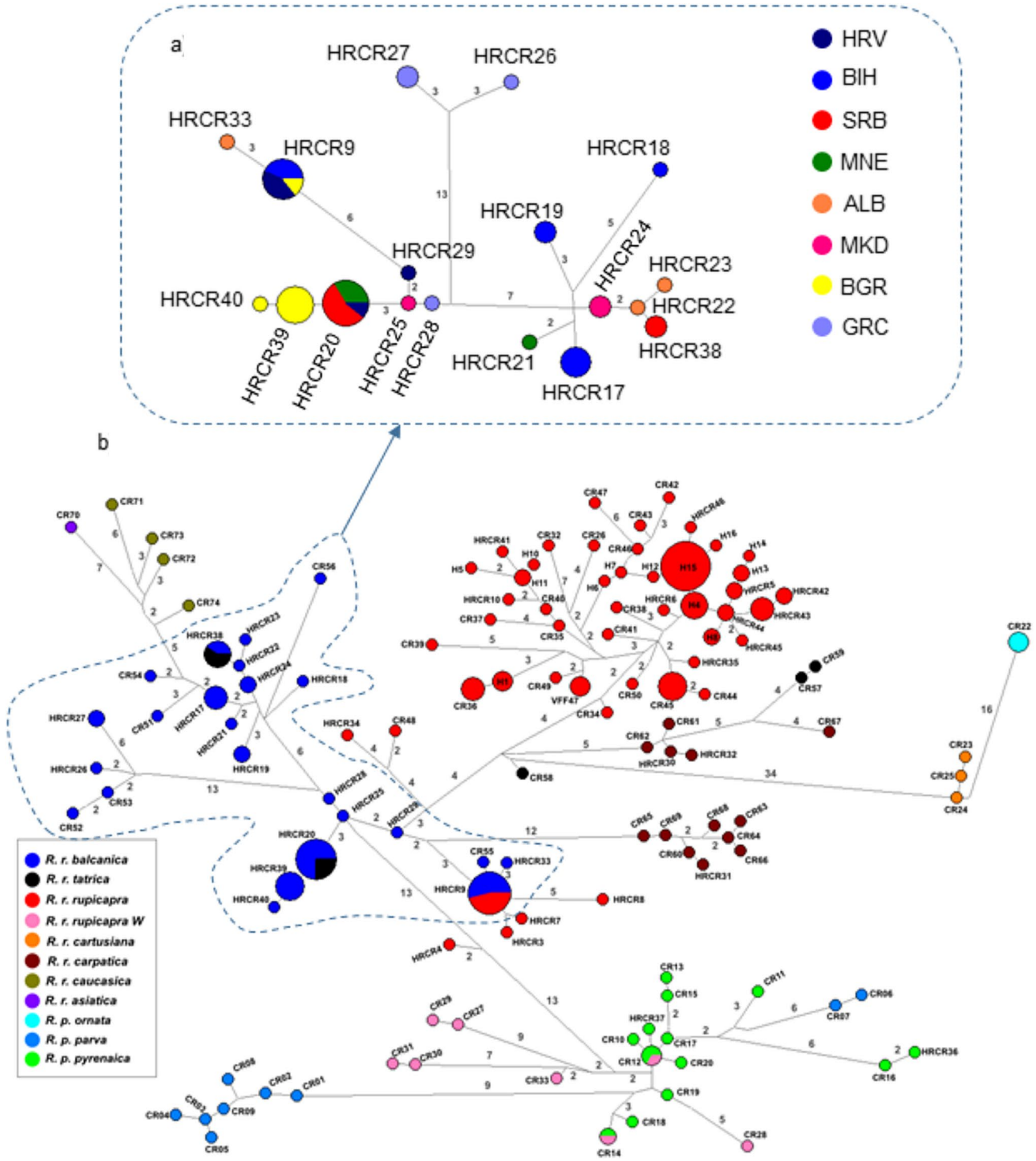

Fig. 3 Median-joining (MJ) networks of the partial CR of mtDNA. The mutations greater than 1 are indicated as grey numbers on the branches, the size of the pie chart represents the number of individuals sharing that haplotype. **a** Median-joining network of Balkan chamois, the different colors represent different countries (populations). **b** Median-joining network of newly generated and GenBank retrieved sequences (209 sequences). Pie chart colors correspond to chamois subspecies. Pie chart labels correspond to the haplotype name deposited in GenBank, haplotypes from HRCR17-HRCR46 are newly described

estimates of genetic diversity compared to the results on the same subspecies by Buzan et al. (2013). These differences in estimates of genetic diversity within the same subspecies could be explained by differences in sample size, the use of different or larger numbers of microsatellites, as well as the representation of different sampling locations. In our study, the average value of observed heterozygosities (0.553) was slightly lower than expected heterozygosity (0.581) indicating a general excess of homozygotes. This might be a consequence of the spatial structuring of populations into subpopulations (Pérez et al. 2002; Buzan et al. 2013). Of particular interest is the low diversity of the BGR population, which comes from the "Izvora State Hunting Reserve" (ISHR) in the western Rhodopes and constitutes a major source population for chamois translocations in Bulgaria. The observed values might be the result of negative fluctuations in the number of chamois in the recent past, the current census size is around 250 individuals and the population is geographically isolated (Markov et al. 2016). According to Hristovich (1939), a total of 150–200 individuals were estimated on the Rhodopes and the total number in Bulgaria was around 1000 chamois. Until the mid-nineteenth century the Balkan chamois had a wider range of distribution in Bulgaria, as it inhabited almost all suitable habitats in the Rila-Rhodopian mountain range. At the end of the nineteenth century, the subspecies disappeared from many of its ranges due to the introduction of long-range rifles (Avramov and Valchev 2010). Markov et al. (2016) suspected that the ISHR population underwent bottlenecks due to overhunting and poaching in its recent history, which might lead to a continuous loss of genetic diversity. The loss of genetic variation is not still seen in the genetic makeup of the BGR population although there are small indications in the observed value of F_{IS} (0.035) showing minor inbreeding, contrary to Markov et al. (2016) expectations of high inbreeding.

Genetic differentiation among Balkan chamois populations

Based on the pairwise F_{ST} values, the SRB population differed significantly from other populations, except MNE. A similar pattern was recorded for the BGR population which differed significantly from the other studied populations (Fig. S.1). These significant F_{ST} values could be explained by populations' isolation within the geographical features of the sampled areas in SRB and BGR (Fig. 1), as they could represent a barrier to gene flow. A genetic differentiation caused by similar factors was found in chamois in other mountain ranges, not only between (Pérez et al. 2002; Crestanello et al. 2009; Rodríguez et al. 2010) but also within subspecies (Crestanello et al. 2009; Buzan et al. 2013; Markov et al. 2016; Papaioannou et al. 2019).

The high genetic differentiation of the BGR population shown by both F_{ST} and STRUCTURE might be a result of the small effective population size in the Rhodopes at the end of the nineteenth century and the multiple genetic bottlenecks experienced by this population (Markov et al. 2016). According to the Action Plan for the Balkan chamois in Bulgaria (Valchev et al. 2006), the genetic differentiation of this population is the result of the absence of natural corridors between populations in West Rhodopes, Rila, Pirin and Tsentralen Balkan, as well as the internal fragmentation at the local population level. In addition to isolation by habitat fragmentation, another factor that could have led to such genetic differentiation could be hybridization with Alpine chamois, since individuals from this subspecies were introduced in 1977 in the Kormisosh hunting reserve (Valchev et al. 2006). The genetic differentiation of the SRB population from neighboring populations was not unexpected, since a similar pattern was observed in other species, including the Dinaric-Balkan wolf population (*Canis lupus*) where two subpopulations were recognized, the "western" subpopulation with individuals from Bosnia and Herzegovina and Croatia and the "eastern" subpopulation with individuals from Serbia and North Macedonia (Djan et al. 2014). Such a structure in the wolf population was attributed to several causes, including bottleneck, different demographic histories of subpopulations, a consequence of differences in hunting pressure, and the possibility that the river Drina acts as a barrier, separating the Peridinaric region (Djan et al. 2014). This river acts as a natural barrier between the Dinarides and the Scardo-Pindic mountain for other species, including the lynx (*Lynx lynx*; Melovski et al. 2012) and wildcat (*Felis silvestris*; Urzi et al. 2021), and its presence might have also affected the Balkan chamois, dividing it into subpopulations. The study on wildcat by Urzi et al. (2021) not only identified a division between western and south-eastern populations but also emphasized the importance of even sampling and avoiding sampling gaps between analyzed areas. It would thus be important to further investigate the Serbian chamois populations, sampling all (or most) of the areas inhabited by the species and combining different molecular markers to clarify the status and population history of chamois in this country to help disentangle the effect of discontinued suitable habitats, historical processes, life histories or environmental barriers on the observed genetic differentiation.

No significant correlation was found between microsatellite genetic distance and geographic distance (Fig. 2). A similar case was found in Alpine chamois (Soglia et al. 2010), where six geographic populations from different sites on the southern slope of the Alps showed no significant correlation of genetic and geographic distances. A significant correlation was found after the removal of the Lombard Prealps population, for which Soglia et al. (2010) hypothesized that it may have suffered from possible impacts of a founder

effect combined with geographic isolation and that different genetic characteristics of the source metapopulation may have disrupted the expected IBD pattern.

Mitochondrial DNA diversity

We observed a relatively high amount of haplotype and nucleotide diversity in the Balkan chamois population ($h = 0.913$; $\pi = 0.031$; Table 2). The Balkan chamois population from the BGR population had very low diversities compared with all other populations, which could be the result of its recent population history with multiple reductions in numbers (Markov et al. 2016). On the other hand, the ALB and GRC populations had high levels of diversity despite a very limited sample size. Here we must take into account the fact that molecular genetic studies of biodiversity that use mtDNA marker variation to characterize the existing genetic diversity of species are particularly sensitive to sample size (Phillips et al. 2018).

The analysis of 44 partial CR sequences of Balkan chamois revealed sixteen private and two common haplotypes (HRCR9 and HRCR20; Fig. 3a), indicating genetic flow among contiguous neighboring populations (SRB, MNE) and the possibility of past translocations, while geographically distant populations (BGR, GRC) were more differentiated. The median-joining network separated GRC haplotypes from haplotypes belonging to other populations (13 mutations). The four Greek Balkan chamois sequences obtained in this study were from the northern Pindus Mountains, where Papaioannou et al. (2019) detected the higher variability among the Greek populations, close to the diversity of the larger populations in the Alps. Similar results were found in the studies of maternally inherited markers in other chamois subspecies (Schaschl et al. 2003; Crestanello et al. 2009; Rodríguez et al. 2009; Rodríguez et al. 2010; Buzan 2013), where substructuring of the maternal gene pool into regional mitochondrial DNA phylogroups with limited gene flow between neighboring populations was observed. Schaschl et al. (2003) showed geographic structuring of mtDNA of Alpine chamois populations in Eastern Alps as a consequence of immigration of chamois from different Pleistocene refugia around the Alps after glacial retreat, and not due to topographic barriers to gene flow. For all population pairs studied in Crestanello et al. (2009), differentiation was always higher for the CR than for the microsatellites, which was to be expected given the higher sensitivity of this locus to genetic drift.

When comparing the Balkan chamois sequences with the dataset containing all other *Rupicapra* spp. (Fig. 3b), one shared haplotype with Alpine chamois and two shared with Tatra chamois were found (HRCR9, HRCR20, HRCR38). The haplotype network grouped Balkan haplotypes from Dinara Mt. in HRV, Rhodope Mt. in BGR, and Prenj Mt. in BIH with Alpine haplotypes from North Velebit Mt. (HRCR9). Similar results were published in Šprem and Buzan (2016), where haplotypes from the Biokovo, Dinara, Velebit and Prenj Mts. grouped with six haplotypes from the Velebit Mt. as a result of past chamois translocations (see Apollonio et al. 2014 for a review). The analysis of the complete mitogenome (Iacolina et al. 2021) revealed the presence of an *R.r. rupicapra* sequence within the *R.r. balcanica* clade. The results reported in the same study showed past reintroductions and translocations in the Northern Dinaric mountains in Croatia. Translocations and genetic introgression might as well explain the presence of the HRCR9 haplotype in the BGR population, since according to Markov et al. (2016) a few individuals of Balkan chamois from the ISHR population may have been introgressed with Alpine genes. An alternative explanation, from the same authors, suggested that the introgression signals might rather reflect shared ancestral polymorphism that might have accumulated through genetic drift.

Based on the median-joining network analysis, Balkan chamois individuals from SRB and MNE populations shared haplotypes with Tatra chamois (HRCR20 and HRCR38; Fig. 3b). The two subspecies were previously described as sister groups (Iacolina et al. 2021) and a similar result was found in the study by Rodríguez et al. (2010), in which the Neighbor-Joining tree, based on combined sequences of different regions of mtDNA, grouped an individual of Balkan chamois close to Tatra chamois. According to the supplementary files of Rodríguez et al. (2010), this one individual that grouped with the Tatra chamois originated from the Serbian Carpathians. The same results were obtained for the mtDNA cytochrome *b* region (Rodríguez et al. 2009), where the Balkan chamois shared the same haplotype with the Tatra, Carpathian, Anatolian and Caucasian chamois. The shared haplotypes between Balkan and Tatra chamois could be explained by possible historical events of introgression between subspecies or shared phylogeny, as previously suggested by Rodríguez et al. (2009).

Conclusions

Results of this study showed the presence of a main genetic cluster comprising most of the Balkan chamois populations, the separation of the BGR chamois into a completely isolated population, whereas the SRB population is only partially isolated as it still shared mtDNA lineages with the MNE chamois. Our results thus reveal that Balkan chamois populations create an archipelago rather than a peninsula as a result of habitat and population fragmentation and an overall reduction in population size due to multiple, non-exclusive factors such as poaching and unsustainable hunting, increased density of infrastructure,

competition with livestock for pastures, predation, and the introduction of other subspecies of chamois, mainly Alpine chamois.

During the last decades, the Balkan chamois has been threatened with extinction in many parts of its distribution range. The introduction of new individuals into small, fragmented populations affected by genetic drift or genetic erosion should be strongly considered. Introductions and translocations, however, should be supported by genetic screening to prevent unwanted negative consequences. We thus recommend a fine scale geographic sampling across the distribution range to detect areas of genetic discontinuity and the identification of (potential) corridors. Overall, long-term (genetic) monitoring and the establishment of MUs is warmly recommended as a basis for sustainable management of these populations and their conservation. The implementation of measures aimed at increasing genetic connectivity, while reducing the risks associated with stochastic effects are needed to improve the sustainability of Balkan chamois populations in the future. Additionally, ancient DNA analysis of archeological and museum samples would help clarifying the subspecies population history and defining its baseline historical genetic diversity.

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Author contributions Nikica Šprem and Elena Bužan conceived and designed the study. Nikica Šprem, Ferdinand Bego, Dragan Gačić, Vladimir Maletić, Georgi Markov, Dragana Milošević, Haritakis Papaioannou collected the samples. Andrea Rezić and Laura Iacolina performed the laboratory analysis. Andrea Rezić, Laura Iacolina, Toni Safner analyzed the data. Andrea Rezić wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read, revised and approved the final manuscript.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval All procedures described in the Materials and Methods section that involve animal experimentation have been approved by the Bioethical Committee for the protection and welfare of animals at the University of Zagreb Faculty of Agriculture. This committee has assessed that the use of Balkan chamois samples was in compliance with the Animal Protection Act (OG 102/17) and the Regulation on the protection of animals used for scientific purposes (OG 55/13).

Consent to participate Research did not involve human participants.

Consent for publication Research did not involve human participants.

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